Successful Use of Oligopeptides as Immunogens in the Preparation of Antisera to Immediate-Early Gene Products of Herpes Simplex Virus Type 1

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SUMMARY

Antisera to two herpes simplex virus-type 1 (HSV-1) immediate-early gene products (IE12 and IE175) have been produced using oligopeptides, constructed on the basis of proposed DNA coding sequences, as immunogens. In both cases the synthetic peptides were linked to bovine serum albumin via an N-terminal tyrosine prior to immunization. Both the IE12 and the IE175 antisera reacted with their respective HSV-1 antigens and with antigens produced by the HSV-1 immediate-early mutant tsK but not with functionally equivalent antigens induced by HSV-2. IE12 induced by tsK was found to have an altered mobility with respect to the wild-type IE12 and its precipitation was accompanied by a second, minor, component of lower mobility. Revertants of tsK gave similar results. Labelling IE12 with a variety of amino acids indicated that the first of three possible initiation codons was used in its translation from mRNA. The results imply that the other initiation codons were not used. Wild-type IE12 is produced for at least 3 h after release of cycloheximide block and appears to be turned over rapidly.

INTRODUCTION

The use of antisera raised against synthetic oligopeptides can provide confirmation of gene sequence analysis as well as linking such analysis to the identification of the gene's protein product. This approach also allows for the production of antisera to minor gene products. Thus, antisera raised to the N- and C-terminal oligopeptides predicted from DNA sequences of simian virus 40 large T antigen immunoprecipitated the antigen (Walter et al., 1980), indirectly confirming that the DNA sequence and the presumed reading frame were correct. Furthermore, antisera to an oligopeptide corresponding to a region of an unknown suspected gene (Sutcliffe et al., 1980) allowed the identification of a hitherto unrecognized gene product (Sutcliffe et al., 1980). The large increase in the number of available sequenced nucleic acids believed to be coding for polypeptides has stimulated interest in the potential of oligopeptide-induced antisera as polypeptide probes. For example, antisera to the hepatitis B envelope protein (Lerner et al., 1981), the VP1 envelope protein of foot-and-mouth disease virus (Bittle et al., 1982) and the influenza virus haemagglutinin (Green et al., 1982) have been raised using oligopeptides as immunogens. A number of other instances have been reported (Sutcliffe et al., 1983).

The production of DNA sequences corresponding to areas within the U region of the herpes simplex virus type 1 (HSV-1) genome (Watson et al., 1981; Murchie & McGeoch, 1982) allows the application of the oligopeptide/immunogen technology to the production of antisera to HSV-coded peptides. We chose to apply the method to two immediate-early gene products, IE175 and IE12. IE175 is a major control protein responsible for the transition from immediate-early to early and late mRNA transcription as well as the switch off of host cell protein synthesis (Parris
et al., 1978; Watson & Clements, 1978; Preston, 1979). IE12, first identified and mapped by Preston (1979) and Marsden et al. (1982), is a polypeptide of unknown function which exhibits a number of atypical features since, unlike other HSV-1 immediate-early polypeptides (IEP), it is neither phosphorylated nor is it transported to the nucleus (Preston, 1979; Marsden et al., 1982). We report here successful production of antisera to both polypeptides, using the sequence data of Murchie & McGeoch (1982). Further characterization of IE12, which may correspond to ICP47 reported by Post & Roizman (1981), using the IE12-specific antisera is also reported.

METHODS

Cells. Propagation of BHK-21 cells (Macpherson & Stoker, 1962) and HFL cells (Haarr & Marsden, 1981) has been previously described.

Virus. HSV-1 strain 17 syn* (Brown et al., 1973), the derived temperature-sensitive mutant tsK syn* (Marsden et al., 1976) and HSV-2 strain HG52 (Timbury, 1971) were used in this study. Revertants of tsK, tsK+ rev 4 and tsK+ rev 5 (Davison, 1981) were kindly provided by our colleague Dr V. Preston.

Radioactive labelling. Confluent monolayers in 30 mm dishes were infected under conditions described in the figure legends, overlaid with Eagle's medium containing 2% foetal calf serum and only one-fifth of the normal concentration of methionine. Cells were labelled in phosphate-buffered saline (PBS) with [35S]methionine (100 μCi/ml), [3H]tyrosine (20 μCi/ml) or [3H]phenylalanine (20 μCi/ml), all from Amersham International (SJ123, TRK530, TRK460 and TRK535 respectively; sp. act. approx. 450 Ci/mmol, 100 Ci/mmol, 20 Ci/mmol and 60 Ci/mmol respectively). Conditions for increased production of immediate-early polypeptides through cycloheximide block have been previously described (Preston, 1979).

Oligopeptides. One IE12 oligopeptide was ordered and purchased from Cambridge Research Biochemicals (Marston, U.K.). Its sequence Tyr-Thr-Asp-Pro-Arg-Asn-Pro-Val-Thr-Arg corresponded to the presumptive C terminus of the IE12 polypeptide (Murchie & McGeoch, 1982) with the addition of an N-terminal tyrosine.

One IE175 oligopeptide was constructed by a continuous-flow solid-phase method (Sheppard, 1983) using a Keiselguhr-supported polydimethylacrylamide resin (Atherton et al., 1981a) and N-fluorenylmethoxycarbonyl protecting groups (Atherton et al., 1981b). The sequence was Tyr-Pro-Ala-Ala-Gly-Thr-Asp-Ala-Gly-Glu-Asp-Ala-Gly which corresponded to the presumptive amino acids from position 65 to 76 as predicted by Murchie & McGeoch (1982), with the addition of an N-terminal tyrosine.

Preparation of antisera. For immunization the two peptides were each linked to bovine serum albumin (BSA) as carrier using bis-diazotized benzidine as described by Bassiri et al. (1979). In the case of the IE12 oligopeptide 2.4 mg were bound to 15 mg albumin (representing a molar ratio of 7:2:1) and for the IE175 oligopeptide approximately 5.4 mg of peptide were bound to 50 mg albumin (a molar ratio of 5:2:1). Antisera were raised in two rabbits with each peptide conjugate. The immunization schedules used a primary injection of 2 mg conjugate in 3 ml of a 1:1 emulsion of antigen in Freund's complete adjuvant injected in six subcutaneous sites followed by a secondary immunisation 14 days later using Freund's incomplete adjuvant. Rabbits were bled after day 21 and given repeat immunizations where necessary to maintain antibody titres. The antisera raised against the IE12 oligopeptide conjugate were designated IE12/75 and IE12/76, and those raised against the IE175 oligopeptide were designated IE175/68 and IE175/69. Anti-BSA was obtained from Miles Laboratories.

Monoclonal antibody 1098, specific for IE175, was prepared essentially as described by Palfreyman et al. (1983), except that tsK-infected 3T12 cells were used as immunogen and SP2/O-Ag14 cells (Shulman et al., 1978) were used as the parental myeloma cell line in the fusion reaction.

Immunoprecipitation. After polypeptide labelling, cells were washed twice with PBS and extracted into either 100 mM-Tris-HCl pH 8.0 containing 10% glycerol, 0.5% Nonidet P40, 0.2 mM-phenylmethylsulphonyl fluoride (Showalter et al., 1981), or processed as described by Preston (1979) into cytoplasmic and nuclear extracts. Cell or cytoplasmic extracts were pre-incubated for 30 min at 4°C with preimmune rabbit serum plus Staphylococcus A (Bethesda Research Laboratories Immunoprecipitin), centrifuged for 5 min on an Eppendorf centrifuge then incubated for 3 h with either oligopeptide-induced antiserum or preimmune rabbit serum. Antibody-polypeptide complexes were separated by binding them to Staphylococcus A. The complex boundaries were washed with 0.5 M-LiCl containing 0.1 M-Tris-HCl pH 8.0 and 1% 2-mercaptoethanol, eluted with 0.125 M-Tris-HCl pH 6.8 containing 2% SDS, 2% glycerol and 5% 2-mercaptoethanol and analysed by SDS-polyacrylamide gel electrophoresis using a 5 to 12.5% gradient gel cross-linked with N,N'-methylenebisacrylamide as described by Marsden et al. (1976).

Polypeptide nomenclature. Polypeptides are labelled according to their apparent molecular weights on gradient polyacrylamide gels as described by Marsden et al. (1976). \( V_{MW} \) 175 (IE175) corresponds to ICP4 (Hones & Roizman, 1974) and \( V_{MW} \) 12 (IE12) may correspond to ICP47 (Post & Roizman, 1981). \( V_{MW} \) 136/143 (E136/143), an early polypeptide, corresponds to ICP6, a probable component of the HSV-1 ribonucleotide reductase (Huszar et al., 1983).
Fig. 1. Comparison of polypeptides produced under immediate-early conditions in cytoplasmic extracts from (a) HSV-1-infected or (c) HSV-2-infected cells, both labelled with $[^{35}S]$methionine from 5 to 7 h after infection, immediately after release of cycloheximide block, or (b) tsK-infected cells, labelled from 5 to 7 h with $[^{35}S]$methionine at the non-permissive temperature. Labelled polypeptides were immunoprecipitated with a control, preimmune rabbit serum (lanes 4) or antisera directed against the IE12 oligopeptide (lanes 2, IE12/75; lanes 3, IE12/76). Lanes designated 1 represent infected cell polypeptide profiles. Numbers to the left of lanes in this and subsequent figures represent the mol. wt. of the immediate-early (175, 110, 63 and 12) or early (E136', 143 labelled 136' in this and subsequent figures) polypeptides of HSV-1 or the equivalent polypeptides of HSV-2. The dot in (b), lane 3 indicates the position of the low intensity, higher mol. wt. minor band found in immunoprecipitates of tsK-infected cells.

RESULTS

Specificity of the IE12 antisera

Two rabbits immunized with the IE12 C-terminal oligopeptide conjugated to BSA each produced antisera that reacted with the HSV-1 IE12 immediate-early gene product [Fig. 1 a, lane 2 (representing antiserum IE12/75) and lane 3 (representing antiserum IE12/76)]. The antisera also reacted with a polypeptide of marginally higher molecular weight produced in cells infected
Fig. 2. (A) Comparison of polypeptides in cytoplasmic extracts from cells infected with HSV-1 under immediate-early conditions and pulsed with [\(^{35}S\)]methionine from 0 to 1 (a), 1 to 2 (b), 2 to 3 (c) or 3 to 4 (d) h after cycloheximide release, or pulsed from 0 to 1 h and chased from 1 to 4 h (e) in methionine-free medium before extraction and immunoprecipitation. Lane designations 1, 3 and 4 are as described in Fig. 1. (B) Polypeptides from cells pulsed with [\(^{35}S\)]methionine for 1 h at 5 h after infection (a, c, e, g, i) or pulsed with [\(^{35}S\)]methionine and chased for 2 h (b, d, f, h, j). (a, b) Mock-infected cells; (c, d) cells infected with HSV-1 under immediate-early conditions; (e, f) tsK-infected cells maintained at the non-permissive temperature; (g, h) and (i, j) cells infected under immediate-early conditions with tsK + rev4 or tsK + rev5 respectively. Lane designations 1, 3 and 4 are again as in Fig. 1. The additional band found in tsK- and tsK revertant-infected cells is again indicated with a dot in lanes 3 of (e), (g) and (i).
with \( tsK \) at the non-permissive temperature (Fig. 1b, lanes 2, 3). A second band of slightly lower mobility was also precipitated from \( tsK \)-infected cells (see also Fig. 2). Revertants of \( tsK \) also produced an altered mobility IE12 polypeptide at the \( tsK \) non-permissive temperature (see Fig. 2). The antisera did not react with the HSV-2 IE12 equivalent polypeptide, the 12.3K immediate-early polypeptide (Fig. 1c, lanes 2, 3). A number of other polypeptides notably IE175 and E136/143 appeared to cross-react with the IE12 antisera (Fig. 1a, lane 3); however, since the control antiserum also showed some apparent reaction with these polypeptides it is probable that their interaction with the IE12 antisera is non-specific. IE118 of HSV-2 also appeared to react non-specifically with both the IE12 and the control antiserum.

**Kinetics of IE12 production**

The production of IE12 together with the other immediate-early polypeptides continued for at least 3 h after the release of cycloheximide block [Fig. 2A(a to d)]. However, turnover of the protein was rapid since on labelling from 0 to 1 h after cycloheximide release and chasing for a further 3 h the polypeptide was very much reduced [Fig. 2A(e)]. The data shown in Fig. 2(B) also indicate that the IE12 equivalent polypeptides produced by both \( tsK \) and its revertants were short-lived and that the minor polypeptide, particularly that of \( tsK^{+}\text{rev4} \), was more rapidly turned over than the major polypeptide. Finally, in Fig. 2B(a, b) it is demonstrated that the IE12 oligopeptide antiserum had no immunoprecipitating reaction with mock-infected cell polypeptides.

**Initiation codon usage**

The deduced mRNA sequence for IE12 of HSV-1 strain 17 (Murchie & McGeoch, 1982) contains potential AUG initiation codons at positions 1, 7 and 15 relative to the first AUG on the IE12 mRNA. Between methionine 1 and methionine 7 there is the only tryptophan residue, between methionine 7 and methionine 15 there is the only phenylalanine and between methionine 15 and the probable stop codon the only tyrosine in the whole predicted amino acid sequence. Immunoprecipitation with the 12K antisera of cells infected in the presence of each of the radio-labelled amino acids demonstrated that they were all incorporated into IE12 (Fig. 3b, c, d, lanes 3). It follows that the first potential initiation codon must be functional. That labelling with each amino acid gave a single discrete band, all migrating with molecular weight similar to that of the \([^{35}S]\)methionine-labelled band, indicates that probably only (or at least predominantly) the first initiation codon is used. The slightly higher molecular weight polypeptide produced by \( tsK \) (compare Fig. 1a, lane 1 with Fig. 1b, lane 1) is probably not produced by altered initiation codon usage since all these amino acids were incorporated into the major polypeptide (Fig. 3f, g, h, lanes 3). The specificity of the immunoprecipitation reaction is demonstrated with the control antiserum used for Fig. 3 (f, g, h) lanes 4.

**Specificity of the IE175 antisera**

Two rabbits were immunized with the IE175 (gene sequence-inferred) oligopeptide conjugate. Both produced antisera that reacted with the 175K immediate-early gene product [Fig. 4a, lane 5 (antiserum IE175/68) and lane 6 (antiserum IE175/69)]. Reaction of the antisera from both rabbits with the polypeptides produced in \( tsK \)-infected cells demonstrated immunoprecipitation of both IE175 and E136/143; moreover, a monoclonal antibody to IE175 gave a very similar precipitation pattern. Neither rabbit's antiserum reacted intertypically with the 182K immediate-early polypeptide which is the IE175 equivalent polypeptide of HSV-2. Some reaction with IE118 can be seen with one antiserum. Since both antisera to IE12 and control antiserum also showed some reaction with IE118 it is probable that this is an artefact of the immunoprecipitation.

The data shown in Fig. 4 suggest that a reaction occurs between IE175 and antisera raised against an IE175 gene sequence-inferred oligopeptide. To confirm that this reaction was specific we attempted to block binding of antisera to the IE175 antigen by pre-incubating the antisera with various amounts of the IE175 oligopeptide. One-hundred \( \mu \text{g} \) or 10 \( \mu \text{g} \) of the oligopeptide almost completely or largely inhibited binding of antisera to IE175 (Fig. 5b, c) with a smaller effect being shown on the precipitation of E136/143 by the antisera. These data also confirm the
Fig. 3. Investigation of initiation codon usage. Cells infected with HSV-1 (a to d) or tsK + rev5 (e to h) under immediate-early conditions were labelled for 1 h (a to d) or 2 h (e to h) after cycloheximide release with [35S]methionine (a, e), [3H]tryptophan (b, f), [3H]phenylalanine (c, g) or [3H]tyrosine (d, h) and immunoprecipitated with control (lanes 4) or IE12 (lanes 3) antisera. Again cytoplasmic extracts were used for immunoprecipitation.
Oligopeptide-induced antisera to HSV-1 IEPP

Fig. 4. Comparison of polypeptides extracted as described by Showalter et al. (1981) from (a) HSV-1-infected or (c) HSV-2-infected cells, both labelled with [35S]methionine under immediate-early conditions, or (b) tsK-infected cells, labelled at the non-permissive temperature (see Fig. 1). Polypeptides were immunoprecipitated with control antisera (lanes 4) or antisera directed against the IE175 gene sequence-inferred oligopeptide (lanes 5, IE175/68; lanes 6, IE175/69). Lanes designated 1 represent infected cell polypeptide profiles, and the lane designated 8 shows immunoprecipitation with IE175-specific monoclonal antibody MA1098.

observation from Fig. 4(a) lanes 5 and 6 that antiserum in lane 5 has a higher avidity for IE175 than antiserum in lane 6. As noted previously the IE12 antisera showed a slight precipitation of IE175 and E136'143, as did an antiserum raised against BSA (Fig. 5d, lane 7). Pre-incubation with 100 μg of the oligopeptide had no effect on the binding of these polypeptides to anti-BSA (Fig. 5d, lane 8).

It seems probable that the precipitation of E136'143 by the IE175 oligopeptide-induced antisera represents a co-precipitation rather than a specific reaction. In an attempt to clarify the situation, immunoprecipitations were carried out in the presence of increasing salt concentrations expected to reduce non-specific protein–protein interactions. However, both IE175 and E136'143 were still present in the immunoprecipitate even when the anti-peptide serum was reacted in 1 M-NaCl.
Fig. 5. Precipitation, with antisera raised against IE175 oligopeptide, of immediate-early polypeptides from cells infected with tsK and labelled with [35S]methionine as described in the legend to Fig. 1. (a) Precipitation under normal conditions (as described in Methods), or in the presence of (b) 100 μg IE175 oligopeptide, (c) 10 μg IE175 oligopeptide or (d) with anti-BSA in the absence (lane 7) or presence (lane 8) of 100 μg IE175 oligopeptide, or (e) after extraction of immediate-early polypeptides into 0.25 M- (lane 5A), 0.5 M- (lane 5B) or 1.0 M- (lane 5C) NaCl. Lane designations 1, 5, 6 and 4 are as in Fig. 4.

DISCUSSION

The two oligopeptides used in this study each induced the production of specific antisera that reacted with the respective authentic HSV-1 immediate-early gene product. The rationales used to decide on the oligopeptide to be constructed differed. The DNA sequence-deduced C terminus of IE12 was chosen for the IE12 oligopeptide since a number of other studies have indicated that C-terminal peptides may be useful as immunogens (Walter et al., 1980, 1982; Bittle et al., 1982) as long as they are sufficiently water-soluble (Lerner et al., 1981). C termini may be conformationally less constrained in the native protein and the addition of a tyrosine to the N terminus allows the oligopeptide subsequently to be linked to the carrier in a way that may mimic its configuration in the native protein (Walter et al., 1980).

When the work reported here was started the C terminus of IE175 was not known for certain; thus, a region of relatively high hydrophilicity was chosen as the potential immunogen. It was not possible to use the deduced region of highest hydrophilicity which would probably correspond to an antigenic determinant (Hopp & Woods, 1981) since the complete DNA sequence had not yet been obtained. The successful production of antisera that specifically immunoprecipitate the IE175 polypeptide supports the impression gained from the literature that oligopeptide immunogens do not necessarily have to correspond to known antigenic determinants.

That both the IE12 and IE175 oligopeptide-induced antisera react with their respective native
proteins confirms that, at least in the regions used as immunogens, the proposed DNA sequences (Murchie & McGeoch, 1982) are correct. Furthermore, confirmation of reading frame usage is provided. The lack of reaction with HSV-2 immediate-early polypeptides indicates that the IE12 and IE175 equivalent HSV-2 polypeptides (IE12:3 and IE182) do not contain the oligopeptide sequences used as immunogens.

The data presented in this paper further characterize IE12. First they confirm its presence in the infected cell cytoplasm and indicate a rapid turnover of IE12 after cycloheximide block and release. The fate of the polypeptide cannot at present be ascertained; it is not apparently found in nuclear extracts (Preston, 1979) and so far we have not been able to identify any breakdown products. (This may, however, merely be a limitation of the presently used SDS–polyacrylamide gel electrophoretic system.) The data confirm the suggestion of Murchie & McGeoch (1982) that, on the basis of Kozak’s (1981) rules, the first available initiation codon is used when IE12 mRNA is translated. The data also confirm previously unreported observations (H. S. Marsden, personal communication) that IE12 produced by tsK has slightly lower mobility than the IE12 of the wild-type virus. The availability of a specific probe for IE12 allows the identification of a second minor IE polypeptide produced by tsK. The origin of this minor polypeptide cannot at present be ascertained; it does not appear to be a stable processing product since it rapidly disappears in pulse–chase experiments. Despite the appearance of an altered 12K polypeptide the temperature-sensitive lesion in tsK has been shown by DNA sequencing of HSV-1 (strain 17), tsK and tsK revertants to be in the IE175 gene (Davison, 1981). However, this does not preclude the presence of a non-lethal mutation in the IE12 gene. So far, we have not found any biological effect ascribable to the altered IE12, and its necessity in in vitro systems has not yet been demonstrated. The availability of monospecific antiserum to IE12 will facilitate further investigation of this polypeptide which is often difficult to distinguish from host cell polypeptides (Marsden et al., 1982), and may allow positive identification of ICP47 described by Post & Roizman (1981) as equivalent to IE12.

The problems associated with the use of the antisera raised against an oligopeptide deduced from the IE175 gene sequence have not been fully overcome. The data indicate that the antisera specifically react with IE175; however, co-precipitation of E136'143 in tsK-infected cells was consistently observed. It seems unlikely that the antisera specifically react with E136'143 since the reaction was only partially inhibited by pre-incubation of the antisera with the oligopeptide, and furthermore, a monoclonal antibody directed against IE175 also appears to immunoprecipitate E136'143 from these cells. It seems most probable that there is some interaction between these proteins under the immunoprecipitation conditions used and this is present under further investigation.

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